New substrates for stem cell control

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New Substrates for Stem Cell Control

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Abstract

The capacity to culture stem cells in a controllable, robust and scalable manner is necessary in order to develop successful strategies for the generation of cellular and tissue platforms for drug screening, toxicity testing, tissue engineering and regenerative medicine. Creating substrates that support the expansion, maintenance or directional differentiation of stem cells would greatly aid these efforts. Optimally, the substrates used should be chemically defined and synthetically scalable, allowing growth under defined, serum-free culture conditions. To achieve this, the chemical and physical attributes of the substrates should mimic the natural tissue environment and allow control of their biological properties. Herein, recent advances in the development of materials to study/manipulate stem cells, both *in vitro* and *in vivo*, are described with a focus on the novelty of the substrates properties, and in application of substrates to direct stem cells.

Introduction

Stem cells offer great opportunities as tools for screening in drug discovery, as surrogates for primary hepatocytes for toxicity assays as well as potential use in regenerative medicine applications, due to their differentiation capacity (1). To realise these potentials, however, it is necessary to develop defined, scalable and reproducible systems for the *in vitro* expansion and controlled differentiation of pluripotent stem cells (PSC).

In vivo, cells reside within and are part of a complex and dynamic three-dimensional (3D) environment – the so-called extracellular matrix (ECM) (Figure 1). This is composed of many components, including numerous proteins, peptides (including growth factors), polysaccharides and proteoglycans. The ECM not only provides cells with structural cues but also facilitates cell–cell interactions, cell adhesion, and allows for cell migration. Furthermore, the soluble factors within the ECM, such a growth factors and cytokines, can also control cell proliferation, differentiation and apoptosis (2,3).

Creating an artificial ECM, for example via the use of feeder layers (e.g. MEFs) or by applying ECM-mimicking substrates (e.g. Matrigel®), for *in vitro* stem cell culture has seen great developments over the past decades, but the use of animal-derived materials poses possible immunogenic responses and innate batch-to-batch variability that could curtail clinical use (4). Therefore, the development of defined, synthetic materials that can support stem cell renewal, maintenance and differentiation is of key importance. Furthermore, the production of phenotypically stable stem cells is of great importance, that potentially could be achieved by mimicking the stem cell niche using synthetic substrates (5).

Conventional 2D cell culture, although facile, is limited by its capacity to mimic the natural 3D environment and culture in 2D can result in altered gene expression, changes in cell metabolism, signalling and morphology compared to cells grown in 3D (6). It should also be considered that *in vivo* stem cells are subjected to multiple

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biophysical, chemical and mechanical cues that help direct cell fate (1,7,8) and it is important to not only understand these cues but to apply them to the material being designed.

Cell surface receptors, such as integrins are critical in cell–matrix interactions. The tripeptide sequence RGD, found in the ECM-protein fibronectin, binds to integrins (9) and has therefore become a common addition to materials to facilitate cell attachment. Other ECM-derived peptide sequences based on, for example laminins and collagen I (10,11), have also been utilised to enhance cell attachment. In addition, the biocompatibility of synthetic substrates can be promoted by the incorporation of other biologically relevant ligands including proteins and growth factors (12). Degradation of the material might also be necessary to allow for cell propagation/movement, and this can be achieved by the use of protease responsive materials that can be broken down by enzymes secreted by the cells thus allowing migration within the material (13).

Other factors to consider are the mechanical properties of materials, since *in vivo* the ECM composition and mechanical properties varies and this plays a role in controlling tissue development (14–16). For example, the ECM of tendon consists largely of collagen I (17), resulting in a high elastic modulus; in contrast the ECM of the inner eye is mainly composed of hyaluronic acid and consequently has a low elastic modulus (18). Thus, by controlling the mechanical properties of synthetic materials, a certain level of cell response can be afforded.

The interaction between the ECM and integrins occurs at the nanoscale and the surface- and nanotopography of a substrate can also be used to modulate cell response (19). Substrate alone, however, is not enough to fully mimic the complex *in vivo* environment. More complex aspects to materials are needed, such as the introduction of gradients (e.g. oxygen, growth factors) (20) and substrate vascularisation (21,22).

This review will focus on advances over the past 5 years on three classes of materials that have been developed for application within the area of stem cell biology, namely protein, peptide and synthetic polymer-based substrates (Figure 2, Table 1), with specific regards to substrate composition, properties and fabrication, as well as their advancements over previously reported materials.

Protein-based substrates for stem cell control

Protein-based substrates (Figure 2a) inherently present multiple cellular signalling molecules that can promote and control cell growth and may result in fewer adverse cell reactions compared to fully synthetic substrates. A fully defined protocol for the differentiation of human embryonic stem cells (hESC) into hepatocytes was reported using recombinant laminin-coated surfaces (23). hESCs were plated on either pure laminin 521 (lam521) or on a mixture of lam111 and lam521 (referred to as lam111) and differentiated to hepatocytes using an 11-day, serum-free protocol (24,25). Both culture substrates produced similar numbers of hepatocytes, but metabolic activity of CYP3A and CYP1A2 was increased on both lam521 and lam111 compared to Matrigel®. Hepatocytes derived on lam111 and lam521 had more organised networks than on Matrigel® and cell phenotype resembling more that of adult hepatocytes, which also was confirmed by whole genome analysis.

Two recombinant proteins, dubbed C7 and P9, a 7-repeat unit of the CC43 WW domain and a 9-repeat unit of a proline-rich peptide, respectively, generate hydrogels as a mixture through specific recognition-binding and have been used in several stem cell applications (26–29). The hydrogels were able to encapsulate adipose-derived stem cells (ADSC) that showed good cell viability in the gels (26). ADSC-loaded hydrogel injection into nude mice resulted in good cell retention and ECM-deposition at the injection site 14-days post-transplantion, in contrast to cells injected without gel encapsulation. In a further study, an 8-arm star-shaped polyethylene glycol (PEG) was conjugated to one repeat unit of the P9 protein (P1). Hydrogels were formed by cross-linking the PEG-P1 conjugate to the C7 protein followed by encapsulation of human induced pluripotent endothelial stem cells (hiPSC-EC) and injection into mice (27). The gels, in combination with vascular endothelial growth factor (VEGF) encapsulation, showed reduced inflammation and demonstrated muscle tissue regeneration. In another study, poly(*N*-isopropylacrylamide) (PNIPAM) was conjugated to the PEG-P1 unit, creating an 8-arm PEG-PNIPAM-P1 component with seven arms conjugated to P1 and one arm to PNIPAM. This component was cross-linked with C7 to form a double-network hydrogel due to the cross-linking between P1 and C7 and the thermal-phase transition of PNIPAM (28). The enhanced mechanical properties of the double network gave higher cell retention at the injection site compared to single hydrogel networks.

Endothelialisation of human mesenchymal stem cell (hMSC) was observed on microcomposites (FN-Au) made from fibronectin (FN) modified gold nanoparticles (AuNPs) (30). Varying the amount of AuNPs in FN-Au composites resulted in different hMSC proliferation rates. Moreover, expression of MMP9 allowed cell migration, while the endothelial marker CD31 was higher in hMSCs grown on FN-Au compared to just FN or TCPS. The FN-Au was therefore proposed as an alternative coating method to increase the biocompatibility of medical devices that encounter blood, e.g. vascular grafts.

A recent advance in mechanotransduction-induced MSC osteogenesis was demonstrated using an in-house built 6 well-plate nanovibrational bioreactor that allowed nanoscale displacements (1000 Hz) of piezo ceramics (31). MSCs were cultured in well-plates coated with a collagen gel that had an elastic modulus a magnitude lower than normally needed for *in vitro* osteogenesis. When applying nanovibrations, MSC osteogenesis was observed without the need for osteospecific media. qPCR analysis of cells cultured within the nanostimulated gels, showed increased levels of several osteogenic markers, including Runt related transcription factor 2 (RUNX2), collagen I, and osteocalcin (OCN), compared with non-vibrated control cultures.

Peptide-based substrates – adaptable materials for cellular modulation

Rather than using full-length proteins, the use of short peptide fragments (Figure 2b) that support stem cell growth is an attractive alternative due to their scalability, their ease of GMP production and synthetic control and affordability. They are also biologically relevant and potentially can provide cells with the cues they need for attachment, signalling, migration, proliferation and differentiation.

Hair follicle regeneration was achieved by encapsulation of stem cells within a self-assembling peptide (32). The peptide Ac-(RADA)$_4$-NH$_2$ (marketed as PuraMatrix®) self-assembles into nanofibers that subsequently give rise to hydrogels. PuraMatrix® was coupled to an RGD sequence, thereby enhancing cell attachment. Mouse neonatal epidermal cells and tissue-derived multipotent skin-derived precursors were mixed into the hydrogel and implanted into nude mice to promote hair follicle regeneration. After three weeks, hair growth was abundant and densely populated hair follicles were observed with higher hair growth compared to cell encapsulation in Matrigel®. Another study with RGD-functionalised PuraMatrix® demonstrated neuronal differentiation of human bone marrow MSCs (rMSC) (33). Human brain-derived neurotrophic factor (hBDNF), known to induce neuron differentiation, was engineered into the rMSCs. Cell growth, proliferation and levels of neuron specific elastase (NSE) and glial fibrillary acidic protein (GFAP) were increased in rMSC encapsulated into RGD-functionalised PuraMatrix® compared to non-modified hydrogels.

An Fmoc-protected tripeptide hydrogel (Fmoc-F$_2$/S), was developed and studied for the effect on cell phenotype in chondrogenesis (34). qPCR indicated high levels of chondrogenic markers RUNX2, SOX9 and type II collagen in human adipose derived pericytes encapsulated into the hydrogels for 7 days, while immunostaining after 28 days showed production of chondrogenic proteins collagen II and aggrecan within the hydrogels. Pericytes were then encapsulated into the hydrogels for 35 days and cultured with and without the presence of chondrogenic induction media. Although the metabolic profile of cells encapsulated within the two sets of hydrogels were broadly similar, some subtle changes were observed, which could account for the resulting difference in cell phenotype. Thus, it is clear that care must be taken in choosing materials and peptides used for stem cell differentiation to control the desired cell phenotype. It should also be noted that the Fmoc group has inherent liability to basic pH, which could perhaps be used to enable cell harvesting, but might also limit longer-term cell culture.

In another study, a peptide hydrogel based on the self-assembling sequence EAK was investigated for hMSC proliferation capacity. The EAK sequence was conjugated to three different molecules (35); (i) A 25-amino acid peptide for cell adhesion based on repeating RGD units ((GRGDSP)$_4$-K); (ii) An h-vitronectin-based peptide (FRHRNRKGK-NH$_2$); (iii) The protein Insulin-like Growth Factor 1 (IGF-1). The N-termini of the RGD-unit, h-vitronectin and IGF-1 were converted to aldehydes via bio-transanimation with pyridoxal phosphate, and these aldehydes then conjugated to a hydroxylamine-conjugated EAK peptide affording oxime cross-linked hydrogels. hMSC adhesion and proliferation was assessed after gel encapsulation (8 days) and showed that

these modified EAK hydrogels increased cell adhesion, proliferation, spreading and elongation compared to the non-conjugated gels.

Peptides have also been incorporated into decellularised tissue scaffolds to improve cell attachment and proliferation. Pig heart valves were decellularised and covalently coupled to a RGD-peptide and/or VEGF via a PEG spacer (36). Endothelial progenitor cells (EPC) were seeded onto the valves and cell adhesion examined. After 8 days of culture, the valves conjugated with both RGD and VEGF demonstrated the highest cell numbers and proliferation rates as determined bya thymidine incorporation assay and qPCR.

Synthetic polymer-based substrates for supporting stem cell growth

The use of fully synthetic polymeric materials (Figure 2c) can offer many advantages since exact control of composition and properties are afforded, while starting materials tend to be inexpensive, making synthesis robust, scalable and affordable. A drawback is the lack of inherent biological activity of synthetic polymers, but by decorating the polymer chains with bioactive molecules such as peptides and growth factors, or even incorporating biologically relevant functional groups, this can be overcome.

Hydrogels with switchable stiffness have been developed by inclusion of a photodegradable unit into PEG hydrogels (37). Upon irradiation (365 nm), the hydrogel's elastic modulus changes from 10 kPa to 2 kPa. hMSCs were initially cultured on very stiff TCPS (3 GPa) or stiff PEG hydrogels (10 kPa), before changing to culturing on softer 2 kPa hydrogels. The activation levels of transcription co-activators YAP/TAZ (regulates cell behaviour in response to mechanical stimulus) and osteogenic marker RUNX2 were dependent on the initial culturing time on the stiffer substrates. This demonstrated that the initial stiff culture conditions had a role in dictating subsequent stem cell response, even after moving to softer substrates, thus indicating stem cells possessed a so-called "mechanical memory".

In a further study of how material mechanical properties affect cell response, spatially controlled PEG hydrogels were created by precisely controlling photodegradation of a stiff (9.6 kPa) hydrogel (38). This gave regularly patterned hydrogels with mechanically softer and stiffer regions. When hMSCs were grown on hydrogels with higher levels of stiffer regions, greater cell spreading and YAP activation were observed along with increased levels of the osteogenic marker alkaline phosphatase (ALP). Changing from a regular to randomly patterned hydrogel, however, resulted in a more rounded cellular morphology and lower YAP activation, while ALP levels decreased and expression of the stem cell marker CD105 increased, indicating lower levels of differentiation. Thus, this gives clues to how spatially controlling the mechanical properties of biomaterials can aid in directing stem cell fate.

A chitosan polymer with nitric oxide (NO) releasing capacity was developed to increase the angiogenic potential of MSC-derived exosomes (39,40). The polymer contained a β-galactose caged NO donor that in response to β-galactosidase released NO. When human placenta-derived MSCs (hP-MSCs) and HUVEC were co-incubated with the NO-releasing polymer, the angiogenesis of HUVEC increased as measured by tube formation assay. Furthermore, an ischemic murine model showed increased VEGF and miR-126 expression in exosomes released from the hP-MSCs by NO stimulus. This was proposed as a novel mechanism for increased angiogenesis, demonstrating the potential of small molecule releasing biomaterials in stem cell applications.

A scalable alternative to adhesion culture for stem cells was achieved by addition of a gellan gum polymer to mTeSR1 media at low concentrations, which inhibited sedimentation of 3D spheres of hPSCs (without gellan gum the spheres sedimented) (41). The suspension culture was scaled-up into gas-permeable membrane bags giving the equivalent number of cells as would have been obtained from seventeen 100 mm dishes.

The capacity of substrate nanotopography to direct stem cell fate has been demonstrated using a polycaprolactone (PCL), imprinted with the surface topography of the marine shell component nacre (42). Nacre is known to induce bone formation in vertebrates, and the mimicking of its nanotopography on PCL was sufficient to induce MSC osteogenesis in comparison to planar PCL scaffolds.

High-throughput screening of materials is important for the efficient identification of an appropriate substrate for a specific cellular application. The Bradley group, along with others, have been instrumental in the development of polymer microarray technology to rapidly identify polymer biomaterials (43,44). Pioneered by

Bradley, ink-jet printing has allowed the synthesis of 100–1000's of cross-linked polymers on a single glass slide (Figure 3) (45,46). The synthesis and screening of some 600 thermally responsive polyacrylate and polyacrylamide-based polymers identified a family of polymers, based on the monomers 2-(acryloyloxyethyl) trimethylammonium chloride (AEtMA-Cl) and 2-(diethylamino)ethyl acrylate (DEAEA) in varying ratios, that supported the long-term growth of hESCs (> 30 passages) and maintenance of the pluripotency markers Oct3/4, Nanog and Sox2 (47). Importantly, thermal detachment of the hESCs was possible by lowering the temperature to 15 °C, enabling passaging without the need of enzymes or chemicals. A similar high-throughput approach identified polymers that supported the long-term culture of primary hMSCs (48) and of mouse embryonic stem cells (mESC) (49).

Another screen of some 7000 different polyacrylates (again using inkjet printing) enabled the identification of a defined substrates supporting hESC growth and maintenance (50). Hit polymers were scaled-up for 35-day incubation and multiple passaging of RH1 cells. Nanog and Oct4 staining showed maintenance of pluripotency on the polymers, which was confirmed by flow cytometry, qPCR and EB differentiation. Similarly, a novel polyurethane was identified via microarray screening and shown to support the growth and self-renewal of the cancer stem cell phenotype of C6 rat glioma stem cells (51).

A step towards high-throughput 3D printing has been the microarray production of PEG hydrogels printed together with mESCs expressing Oct4-GFP (52). By changing the stiffness of the PEG hydrogels, adding in MMP sequences, ECM components, cell–cell interaction mediating molecules and soluble factors, plus varying the cell seeding density, over 1000 different microenvironments were created in a 1,536 well-plate format. Recently, similar PEG hydrogels have been used for iPSC reprogramming (39), intestinal organoid maintenance (40), ESC neural tube formation (41) and pancreatic progenitor cells (53–56).

Conclusion

Stem cells hold great potential for a range of purposes, from increasing the value of functional cell-based assays and *in vitro* pharmaceutical testing to revolutionising regenerative medicine by providing novel treatments for a plethora of diseases and conditions. As highlighted here, the rapid development of substrates to support stem cell growth, maintenance and differentiation can greatly aid in these pursuits. As the understanding of stem cell biology expands, the design and engineering of substrates will be enhanced, particularly with regards to including control over topography, chemical and mechanical properties, biological factor inclusion and modulation of degradation/remodelling properties. In addition, transitioning from 2D to 3D substrates will create better *in vivo* mimics, although with the added hurdle of more complicated analysis. However, advances in biofabrication and the use of high-throughput analysis systems are sure to alleviate this problem. Finally, these efforts require knowledge and skills from multiple disciplines, covering biology, medicine, chemistry, engineering and physics to name but a few. It is only via collaborative efforts that regenerative medicine will ever be able to truly deliver and enable the translation of stem cells and accompanying partner materials into clinical applications.

Additional Information

Authors' Contributions
All authors contributed equally to the manuscript preparation

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We have no competing interests

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Tables

Table 1: Recent advances in material development for stem cell applications

Material source	Stem cell application
Protein	
Albumin (57)	MSC osteogenesis
Alginate (58)	hPSC switch from self-renewal to differentiation
Collagen (59)	MSC chondrogenesis
Collagen nanovibrational bioreactor (31)	MSC osteogenesis
Collagen-RGD (60)	MSC articular cartilage formation
Elastin with citric acid (61)	MSC osteogenesis
ELP, ELP-HA (62,63)	ADSC, MSC
Fibrin-IKVAV (64)	Neural SC differentiation
Fibronectin-Au (30)	MSC endothelialisation
Heparin (65)	hPSC expansion
Hyaluronic acid (66)	MSC chondrogenesis
Laminin521 and Laminin111 (23)	hESC to hepatocyte
Recombinant protein hydrogels (26–29)	hiPSC-EC, ADSC,
Peptide	
Au-SAM with RGD (67)	MSC differentiation capacity
EAK (35)	MSC proliferation
Fmoc-F_2/S (34)	Pericyte chondrogenesis
RADA-PRG (modified PuraMatrix®) (32,33)	Mouse neonatal epidermal cells, rMSC neurogenesis
RGD on decellularised pig heart valves (36)	EPC proliferation
Synthemax® (68)	iPSC expansion and differentiation
Polymer	
Enzymatically-responsive PEG (52–56,69)	iPSC, hESC, mESC, MSC, organoids, mouse pancreatic progenitor
Gellan gum (41)	hPSC 3D spheres
Methycellulose (70)	MSC chondrogenesis
Nanotopographically imprinted PCL (42,71)	MSC maintenance and osteogenesis
NO-releasing chitosan (39)	hP-MSC angiogenic potential
P(PEGMEMA-r-GMA-r-VDM) (72)	MSC proliferation
PEG with RGD and MMP tethering (73,74)	MSC migration
Photodegradable PEG (37,38)	MSC ”mechanical memory”

Polyacrylate/acrylamide thermoresponsive hydrogel (47–49)	hESC, MSC, mESC
Polyacrylate/polyurethane (50,51,75)	hESC, MSC, C6 rat GSC
Polypyrrole (76)	MSC osteogenesis
Polystyrene TopoChip (77)	iPSC proliferation
Polyurethane (78–81)	hESC-derived HE, HPC, iPSC-derived hepatocytes, H9
Ternary polymer blends (82,83)	STRO-1$^+$ skeletal SC, foetal skeletal SC

Figure and table captions

Table 1: Recent advances in material development for stem cell applications

Figure 1. Representation of the ECM and its functions, which in combination with cell signalling directs stem cell fate.

Figure 2. The substrates covered in this review for stem cell growth and regulation and some of their pros and cons.

Figure 3. Principle of ink-jet printing for the formation of polymer microarrays.

Figures

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